Characterization, regulation, and phylogenetic analyses of the *Penicillium griseoroseum* nitrate reductase gene and its use as selection marker for homologous transformation

Jorge Fernando Pereira, Marisa Vieira de Queiroz, Francis Júlio Fagundes Lopes, Rodrigo Barros Rocha, Marie-Josée Daboussi, and Elza Fernandes de Araújo

Abstract: *Penicillium griseoroseum* has been studied because of its efficient pectinases production. In this work, the *Penicillium griseoroseum* nitrate reductase gene was characterized, transcriptionaly analyzed in different nitrogen sources, and used to create a phylogenetic tree and to develop a homologous transformation system. The regulatory region contained consensus signals involved in nitrogen metabolism and the structural region was possibly interrupted by 6 introns coding for a deduced protein with 864 amino acids. RT–PCR analysis revealed high amounts of *niaD* transcript in the presence of nitrate. Transcription was repressed by ammonium, urea, and glutamine showing an efficient turnover of the *niaD* mRNA. Phylogenetics analysis showed distinct groups clearly separated in accordance with the classical taxonomy. A mutant with a 122-bp deletion was used in homologous transformation experiments and showed a transformation frequency of 14 transformants/μg DNA. All analyzed transformants showed that both single- and double-crossover recombination occurred at the *niaD* locus. The establishment of this homologous transformation system is an essential step for the improvement of pectinase production in *Penicillium griseoroseum*.

Key words: nitrate reductase, nitrogen metabolism, *Penicillium griseoroseum*, phylogenetic analysis, homologous transformation.

Résumé : Notre groupe étudie *Penicillium griseoroseum* à cause de sa production efficace de pectinases. Nos travaux ont porté sur la caractérisation du gène de la nitrate réductase de *P. griseoroseum*, de même que sur son analyse transcriptionnelle en présence de diverses sources d'azote, et celui-ci a été utilisé afin de concevoir un arbre phylogénétique et un système de transformation homologue. La région régulatrice renfermait des signaux consensus impliqués dans le métabolisme de l'azote et la région structurale est potentiellement interrompue par six introns et coderait une protéine déduite de 864 acides aminés. Une analyse par RT–PCR a révélé de fortes quantités de transcrits de *niaD* en présence de nitrate. La transcription a été réprimée par l'ammonium, l'urée et la glutamine, ce qui démontre un taux de renouvellement efficace de l'ARNm de *niaD*. Les analyses de phylogénétique ont permis de distinguer des groupes nettement séparés conformément avec la taxonomie classique. Un mutant contenant une délétion de 122 pb a été employé dans les expériences de transformation homologue avec une fréquence de transformation de 14 transformants/μg d'ADN. Tous les transformants analysés ont démontré que des recombinaisons à enjambement simple et double se sont produites dans le locus *niaD*. La conception de ce système de transformation homologue représente une étape essentielle dans le programme d'amélioration de la production de pectinases chez *P. griseoroseum*.

Mots clés : nitrate réductase, métabolisme de l'azote, *Penicillium griseoroseum*, analyses phylogénétiques, transformation homologue.

[Traduit par la Rédaction]

Introduction

In filamentous fungi, the genes related to the catabolism of different nitrogen sources are repressed by ammonium and glutamine. In *Aspergillus nidulans*, global control is mediated by the positive-acting AREA protein and by the negative-acting NMR protein. AREA contains one single GATA-binding domain (Kudla et al. 1990; Langdon et al. 1995), and the corresponding gene in *Neurospora crassa* is called *nit-2*. The extreme C-terminal residues and the DNA-

Received 10 February 2004. Revision received 27 May 2004. Accepted 30 June 2004. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 6 January 2005.

J.F. Pereira, M.V. de Queiroz,[1] F.J.F. Lopes, R.B. Rocha, and E.F. de Araújo. Departamento de Microbiologia/BIOAGRO, Universidade Federal de Viçosa, 36.570-000, Viçosa-MG, Brazil.
M.-J. Daboussi. Institut de Génétique et Microbiologie, Université Paris-Sud, 91405 Orsay, France.

[1]Corresponding author (e-mail: mvqueiro@mail.ufv.br).

 doi: 10.1139/W04-081

binding domain of AREA and NIT-2 are highly conserved. The NMR protein is able to bind to a small region of the DNA-binding domain and to the carboxy-terminal region of *Neurospora crassa* NIT-2 protein (and possibly of AREA), blocking the function of NIT-2. Glutamine is possibly the signal for this binding (Xiao et al. 1995). Therefore, AREA is unavailable and the cell utilizes ammonium and glutamine, preferentially. An excellent secondary nitrogen source used by many fungi is inorganic nitrate. In most filamentous fungi, the expression of the nitrate reductase gene depends on the absence of ammonium and glutamine and the presence of nitrate as inducer. In this situation, a specific factor called *nirA* in *Aspergillus nidulans* and *nit-4* in *Neurospora crassa* is necessary in addition to the global regulation factor (Caddick et al. 1994; Marzluf 1997). The only reported exception to this rule is *Hebeloma cylindrosporum* in which the transcriptional regulation of nitrate assimilation genes is under ammonium repression but does not require nitrate for induction (Jargeat et al. 2000, 2003).

The analysis of nitrate reductase gene sequences in several filamentous fungi reveals the presence of from 0 to 12 introns (Banks et al. 1993; Levis et al. 1997*a*; Jargeat et al. 2000). In *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium chrysogenum*, 6 introns with different sizes were found, but in the same positions (Johnstone et al. 1990; Unkles et al. 1992; Kitamoto et al. 1995; Haas et al. 1996). Moreover, at least for *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus fumigatus*, and *Penicillium chrysogenum*, the nitrate and nitrite reductase genes are clustered and transcribed in opposite directions from an intergenic region (Johnstone et al. 1990; Unkles et al. 1992; Haas and Marzluf 1995; Chang et al. 1996; Amaar and Moore 1998).

In addition, genes enconding nitrate reductase have been cloned to establish transformation systems. This system presents advantages, such as easy isolation of spontaneous mutants, reduced possibility of secondary mutations, and a dispensable mutant phenotype. Homologous transformation systems, based on the complementation of nitrate reductase mutations, have been developed for several filamentous fungi. These studies report an increased transformation frequency, compared with heterologous systems, except for *Stagonospora* (*Septoria*) *nodorum* (Cutler et al. 1998), whose frequency remained unaltered. Furthermore, a high homologous integration frequency is reported for *Aspergillus oryzae*, *Botrytis cinerea*, and *Stagonospora nodorum* (Unkles et al. 1989; Levis et al. 1997*a*; Cutler et al. 1998).

Penicillium griseoroseum has been studied by our group because of its efficient pectinolytic enzyme production, with the goal of using these enzymes in industrial processes. Queiroz et al. (1998) have described a heterologous transformation system for *Penicillium griseoroseum*, using the *Fusarium oxysporum* nitrate reductase gene, with a low transformation frequency and with most of the integrations in ectopic sites. In the present study, the *Penicillium griseoroseum* nitrate reductase gene (*niaD*) was characterized, transcriptionally analyzed in different nitrogen sources, and used to create a phylogenetic tree and to develop a homologous transformation system. We isolated the mutant PG63 with a 122-bp deletion in the *niaD* gene, which has ideal characteristics as a recipient strain in transformation experiments. Homologous transformation shows homologous integration in all analyzed transformants. For the first time, we report the complementation of a *nirA* mutant in *Penicillium* with the *Aspergillus nidulans nirA* gene.

Materials and methods

Fungal strain and isolation of chlorate-resistant mutants

The *Penicillium griseoroseum* strain (CCT 6421) was isolated by Dr. J.J. Muchovej, Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa-MG, Brazil. The isolation of chlorate-resistant mutants was carried out exactly as described by Unkles et al. (1989). The characterization of chlorate-resistant mutants was based on Cove's (1979) method in relation to their ability to grow in different nitrogen sources. The phenotype symbols used in *Aspergillus nidulans* were adapted for comparable phenotypes in *Penicillium griseoroseum*.

Plasmids

Plasmid pUES3.2, which contains a 3165-bp *Eco*RI/*Sal*I fragment corresponding to a large part of the coding region of the *Penicillium chrysogenum niaD* gene, was kindly provided by Dr. Hubertus Haas, Institut für Mikrobiologie, Universität Innsbruck, Innsbruck, Austria. Vector pNIRA2, detailed by Burger et al. (1991), contains the *nirA* gene from *Aspergillus nidulans* and was kindly provided by Dr. Maribel Isabel Muro-Pastor, Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas de la Isla de la Cartuja, Espanha.

Isolation, sequencing, and sequence analyses of the *Penicillium griseoroseum niaD* gene

A λEMBL3 genomic library of *Penicillium griseoroseum* was constructed as described previously (Ribon et al. 1999). The 3165-bp fragment, containing a large part of the *niaD* structural region of *Penicillium chrysogenum*, was used to probe the phage library of genomic DNA under low-stringency conditions. Recombinant phage DNA was extracted and fragments of the selected lambda clones were subcloned according to standard procedures (Sambrook et al. 1989). Inserts of interest were subcloned into the pBluescript KS+ (Stratagene) plasmid. Sequencing of DNA was carried out by the dideoxy chain termination method (Sanger et al. 1977) using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Resulting DNA sequences were used to search databanks using the BLAST Service Network at NCBI (www.ncbi.nlm.nih.gov/BLAST). Subsequent DNA and protein sequence analyses were carried out with the CLUSTAL W program (Thompson et al. 1994). The nucleotide sequence data reported here appear in the EMBL nucleotide sequence database under accession No. AY255803.

Phylogenetic analysis

Additional nitrate reductase protein sequences of 20 filamentous fungi, 2 yeasts, 3 plants, and 1 oomycete were obtained from GenBank and used in the phylogenetics analysis (GenBank sequence numbers in parentheses): *Aspergillus fumigatus* (AF336236), *Aspergillus nidulans* (M58291),

Aspergillus niger (M77022), *Aspergillus oryzae* (D49701), *Aspergillus parasiticus* (U38948), *Aspergillus thaliana* (P11832), *Beauveria bassiana* (X84950), *Botryotinia fuckeliana* (U43783), *Fusarium oxysporum* (Z22549), *Gibberella fujikuroi* (X90699), *Hebeloma cylindrosporum* (AJ238664), *Leptosphaeria maculans* (U04445), *Metarhizium anisopliae* (AJ001141), *Monascus purpureus* (AB046444), *Neurospora crassa* (X61303), *Neurospora tabacum* (X14059), *O. sativa* (P16081), *Pichia angusta* (P49050), *Pichia anomala* (AF123281), *Penicillium chrysogenum* (U20779), *Phytophthora infestans* (U14405), *Stagonospora nodorum* (AJ009827), *T. borchii* (AF533362), *Ustilago maydis* (AJ315577), *V. carteri* (P36841), and *V. fungicola* (AAO63560). Protein alignment was performed using the CLUSTAL W program and were manually adjusted. The final alignment, which excludes the extremity of the fragments and hypervariable regions, was saved as a PAUP file. Three internal positions could not be aligned and were ignored by equating them to missing data. Parsymony analyses were conducted in PAUP* version 4.0 (Swofford 2003) to compare 342 parsimony-informative characters in the NIAD protein. This program was used to process a heuristic search and bootstrap analysis using 1000 replicates. Four trees equally parcimoneous were found, and a consensus tree was constructed.

RT–PCR analysis of *niaD* expression under different nitrogen sources

All nitrogen sources were prepared in a 2.5 mol/L stock solution, filter-sterilized, and added to the minimal medium (Pontecorvo et al. 1953) after autoclaving. A volume of 100 mL of liquid minimal medium supplemented with 25 mmol/L each of ammonia, urea, glutamine, and nitrate was inoculated with a spore solution for a final concentration of 1×10^6 spores/mL and incubated at 25 °C at 150 r/min. After 36 h, mycelia grown in ammonia or urea were collected and quickly frozen in liquid nitrogen. Nitrate-grown mycelia were collected at 0, 15, and 60 min after the addition of glutamine stock solution up to a final glutamine concentration of 25 mmol/L and quickly frozen. Mycelia grown in glutamine were filtered, washed with sterile water, and transferred onto a minimal medium containing 25 mmol/L nitrate and then collected at 0, 15, and 60 min after transfer and quickly frozen. The total RNA was extracted by the method described in Deeley et al. (1977) with few modifications. In a 20-μL reaction mixture, 1 μg of total RNA was reverse-transcribed at 42 °C for 90 min using the Reverse Transcription System (Promega) according to the manufacturer's instructions. The *niaD*-specific PCR products for mRNA (1778 bp) and for total DNA (2123 bp) were amplified by the primers PC1 (5′-AAGGCACACCGGATAACCAT-3′) and T2 (5′-TAGGCCGAGTGTCTGCTTGT-3′). Five microlitres of the RT reaction were used in a PCR reaction. The PCR program was as follows: 40 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 2 min (for the amplifications with the cDNA), or 72 °C for 3 min (for amplifications with total DNA). A γ-actin specific PCR product of approximately 500 bp was amplified as a control using the specific primers Act F (5′-ACACCTTCTACAACGAGCTG-3′) and Act R (5′-GGAAGCTCGTAGGACTTCTC-3′). The entire volume of each amplification reaction (25 μL) was electrophoresed in a 1.5% agarose gel.

Preparation and transformation of protoplasts

Protoplast preparation was based on the method described by Ballance and Turner (1985) with some modifications. Complete liquid medium (Pontecorvo et al. 1953) was inoculated with 1×10^7 spores of the mutants PG4 (*nirA*$^-$), PG18 (*nirA*$^-$), and PG63 (*niaD*$^-$) and incubated at 25 °C at 150 r/min for 14–18 h. Mycelia were collected by filtration, mixed with 15 mg of Novozym 234 and 5 mL of 0.8 mol/L KCl (pH 5.8), and incubated at 30 °C for 2–3 h under gentle shaking. Protoplasts were filtrated, washed 3 times in STC (1 mol/L Sorbitol, 50 mmol/L $CaCl_2$, and 100 mmol/L Tris–HCl) by centrifugation at 2000*g* at 4 °C for 15 min, and resuspended in a suitable volume for a final concentration of 10^8 protoplasts/mL. Transformation was based on Yelton et al. (1984) and Ballance and Turner (1985) and exactly as described by Queiroz et al. (1998). Protoplasts (1×10^7) were mixed with 50 μL of PEG–$CaCl_2$ solution (60% PEG 6000 and 50 mmol/L $CaCl_2$) and 5 μg of plasmid DNA (pNIRA2 or pNPG1). The mixture was incubated on ice for 20 min before the addition of 500 μL of the same PEG–$CaCl_2$ solution. After 20 min at room temperature, the preparation was centrifuged for 5 min at 12 000*g* and resuspended in 0.5 mL of STC. The protoplasts were plated on selective minimal medium containing nitrate as sole nitrogen source and 0.56 mol/L sucrose as osmotic stabilizer. Negative controls were carried out with protoplasts that were not mixed with the plasmid DNA. The plates were incubated at 25 °C for 5–6 d.

DNA analyses of *niaD*$^-$ mutants and *niaD*$^+$ transformants

The total DNA of the wild-type 21 *niaD*$^-$ mutants and 5 randomly chosen transformants were extracted as described by Specht et al. (1982). Size separation of the total DNA (3 μg) digested with the restriction enzyme (*Eco*RV and *Acc*I for *niaD*$^-$ mutants and *Sph*I for the *niaD*$^+$ homologous transformants) was performed in a 0.7% agarose gel. Transfer onto a Duralon-UV™ membrane (Stratagene) was performed according to general protocols (Sambrook et al. 1989). Hybridizations were carried out at 60 °C using the plasmid pUES3.2 as probe for the *niaD*$^-$ mutants and *niaD*$^+$ homologous transformants. The DNA fragment containing the deletion in the PG63 mutant *niaD* gene was obtained by amplification with the primers T1 (5′-CCGGAGGAGCGATCAGTATT-3′) and T2 (5′-TAGGCCGAGTGTCTGCTTGT-3′). The PCR product was subcloned using pGEM-T Easy (Promega) and sequenced as described above.

Results

Cloning of *Penicillium griseoroseum* nitrate reductase gene

Two lambda clones were isolated from the *Penicillium griseoroseum* genomic library. A 6.1-kb *Kpn*I fragment that hybridized with the probe was cloned to create the pNPG1 plasmid. This plasmid was partially sequenced by subcloning and by using specific primers for the *niaD* sequence (Fig. 1). The structural region of the *Penicillium griseoroseum niaD* gene contains 2940 bp. The *niaD* gene possibly pos-

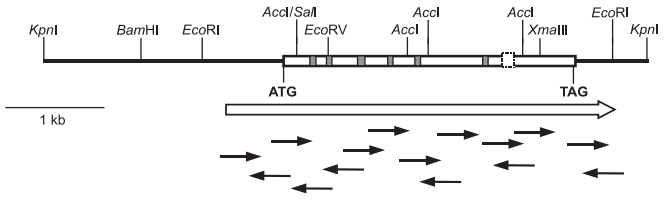


Fig. 1. Sequencing strategy and partial restriction map of the 6.1-kb *Kpn*I fragment of the pNPG1 plasmid containing the *Penicillium griseoroseum* nitrate reductase gene. The bar represents the structural region of the *niaD* gene. Solid boxes indicate putative intron positions and open boxes the position of deleted regions in mutant PG63. The large arrow indicates the transcription direction. The small arrows represent localization and direction of oligonucleotides used in the sequencing reactions. See nucleotide sequence at GenBank (accession No. AY255803).

sesses 6 introns with 5′ and 3′ sequences (GTR and AG) and internals sequences (such as TAAG, TAAC, and CAAT), involved in the removal process, similar to the consensus sequence described by Gurr et al. (1987). These putative introns are 60, 54, 72, 51, 55, and 53 bp in length. The introns II, III, IV, and VI did not present any similarity to the introns of other filamentous fungi, except in the regions necessary for their removal, although introns I and V showed a similarity of 88% and 81%, respectively, to introns I and V of the *Penicillium chrysogenum niaD* gene.

The 5′ region next to the possible start codon contains a series of possible promoter elements. These elements include 3 possible TATA box regions at –52, –109, and –261 bp (in relation to the proposed ATG) and 3 possible CAAT box regions at –44, –98, and –170 bp. The region around the start codon (CCATC**ATG**GC) shows a purine (adenine) in the –3 bp position, identified as important for translational activity by Gurr et al. (1987). Four GATA and 4 TATC sequences are found in this promoter region. These *cis*-elements are binding sites of the general positive regulatory AREA in *Aspergillus nidulans* and NIT-2 in *Neurospora crassa* (Caddick et al. 1994; Marzluf 1997). In *Penicillium griseoroseum*, a possible binding region of the specific regulatory protein NIRA (CTCCGTGG) is localized at –483 bp, identical to the consensus for *Aspergillus nidulans* (CTCCGHGG) described by Punt et al. (1995). The promoter region of the *Penicillium griseoroseum niaD* gene presents a similarity of 12.5% to a corresponding region of *Penicillium chrysogenum*. In the 3′ nontranslated sequence of the *Penicillium griseoroseum niaD* gene, no site identical to the consensus (AATAAA), described by Gurr et al. (1987), could be identified, although this region shows AT-rich sequences. A possible polyadenylation site (AAAAATA) was observed at 71 bp of the possible stop codon.

The removal of possible introns from the *Penicillium griseoroseum niaD* gene revealed an open reading frame of 2595 nucleotides encoding a protein of 864 amino acid residues with a deduced molecular mass of 96.7 kDa. The deduced amino acid sequence presents the following homologies with others nitrate redutases: 92% with *Penicillium chrysogenum*, 76% with *Aspergillus niger*, 74% with *Aspergillus oryzae*, 71% with *Aspergillus parasiticus*, 62% with *Leptosphaeria maculans* and *Phaeospaeria nodorum*, 58% with *Botryotinia fuckeliana*, 57% with *Beauveria bassiana*, *Fusarium oxysporum*, *Gibberella fujikuroi*, and *Metarhizium anisopliae*, 54% with *Neurospora crassa*, 44% with *Hebeloma cylindrosporum*, 40% with *Ustilago maydis*, 40% with *Zea mays*, 39% with *Spinacia oleracea*, and 38% with *Glycine max* and *Phaeseolus vulgaris*, among others.

Conserved regions that correspond to the molybdenum-cofactor-, heme-, and FAD-binding domain, essential for the activity of nitrate reductase, were identified in the NIAD protein of *Penicillium griseoroseum* (Fig. 2). The molybdenum-cofactor-binding domain is interrupted by the first 5 introns. In this domain, amino acid Cis^{149} was found, which corresponds to Cis^{150} in *Aspergillus nidulans* and is essential for the binding of the molybdenum cofactor in studies on directed mutagenesis (Garde et al. 1995). The region related to the heme group binding is interrupted by intron 6. In this domain, a histidine in position 547 is highly conserved among several analyzed nitrate reductase proteins, which was also demonstrated for *Penicillium griseoroseum*. The nucleotide sequence that corresponds to the FAD domain is not interrupted by any intron and presents conserved amino acids in *Aspergillus nidulans*, Trp^{618}, His^{654}, and Gli^{721}, which do not seem to be essential but important for protein activity (Garde et al. 1995).

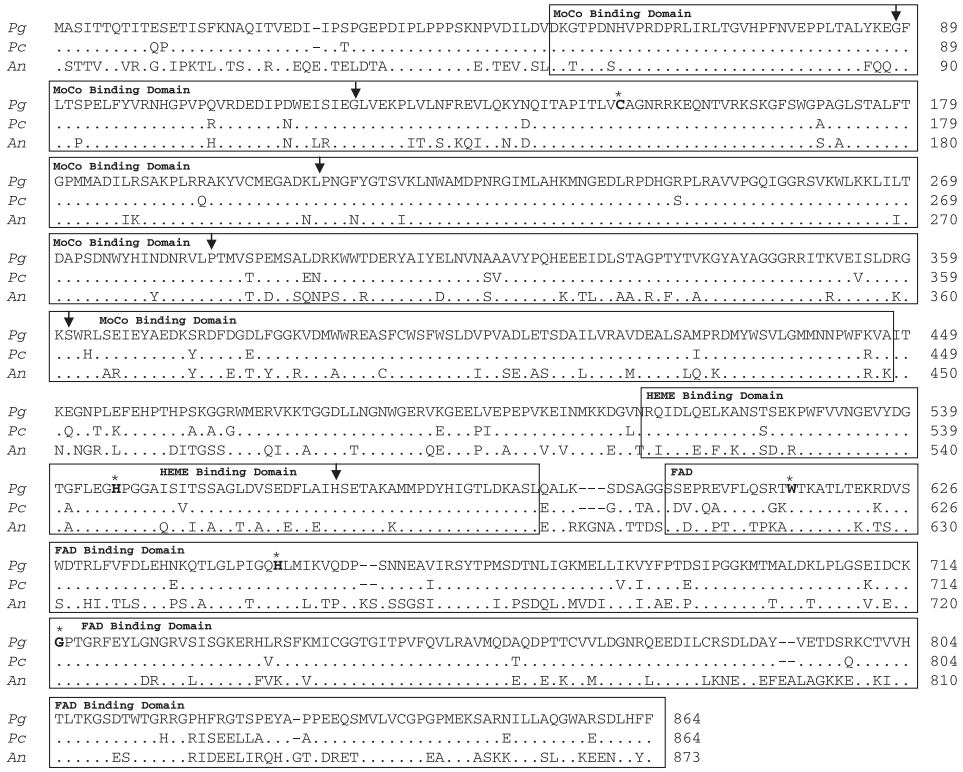
Expression of the *Penicillium griseoroseum* nitrate reductase gene under different nitrogen sources

The *niaD* gene transcription was activated when the glutamine-grown mycelia were transferred to a medium with nitrate as the only nitrogen source (Fig. 3). The DNA fragment amplified from the cDNA has the expected size of the structural region. This shows the presence and size of the possible introns in the *Penicillium griseoroseum niaD* gene. After 15 min of induction with nitrate, a great quantity of amplified cDNA was detected. After 60 min, a quantity similar to the maximum (36 h of activation or 0 min in glutamine) was observed. Fifteen minutes after the nitrate-grown mycelia had been exposed to glutamine, there was a considerable reduction in the amplified *niaD* transcript quantity, and after 60 min, it was near the basal level. Interestingly, a small quantity of amplified cDNA was detected in the cultures supplemented with ammonium, glutamine, or urea.

Phylogenetic analysis of *Penicillium griseoroseum* nitrate reductase protein

After the characterization of the *Penicillium griseoroseum niaD* deduced protein, a phylogram was constructed as a rooted phylogenetic tree (Fig. 4). Distinct groups clearly separating fungi, plants, algae, and the oomycete are observed. In the cluster containing yeasts and filamentous fungi, there is a tendency of grouping species based on the order of the classical taxonomy and by the number of introns in the *niaD* gene. *Aspergillus* sp., *Penicillium* sp., and *Monascus purpureus* belong to Order Eurotiales of Phyllum Ascomycota, have 6 introns in the *niaD* gene, and were clustered together. *Leptosphaeria maculans* and *Stagonospora nodorum* belong to Order Pleosporales and have 4 introns in the *niaD* gene. In the cluster containing the species of Order Hypocreales, the *niaD* gene is interrupted by only 1 intron. The yeasts clustered together belong to Order Saccharomycetes and do

Fig. 2. Deduced amino acid sequence of *Penicillium griseoroseum* NIAD protein (*Pg*) aligned with homologous polypeptides from *Penicillium chrysogenum* (*Pc*) (GenBank U20779) and *Aspergillus nidulans* (*An*) (GenBank M58291). Dots represent amino acids present in *Penicillium griseoroseum* protein; hyphens show gaps. The 3 functional domains (molybdenum cofactor (MoCo), heme, and FAD) are indicated by boxes. Arrows indicate intron positions. Amino acids with asterisks indicate conserved residues that have proved to be important in directed mutagenesis experiments for the *Aspergillus nidulans* NIAD protein (Garde et al. 1995).



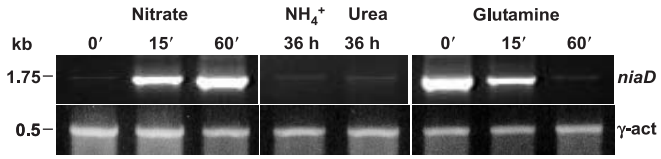
not have introns. This tendency in cluster species with similar numbers of introns is not true for basidiomycetes where there is a great difference of 0–12 introns for *Ustilago maydis* and *Hebeloma cylindrosporum*, respectively. The consistency index for this tree was 0.6105.

Isolation of nitrate reductase mutants

After 15 d of growth at 25 °C, *Penicillium griseoroseum* chlorate-resistant colonies were isolated. All of the 52 chlorate-resistant colonies grew in ammonium, glutamate, and hypoxanthine, whereas 21 failed to grow in nitrate and 14 did not grow in nitrate and nitrite. Since growth was observed in hypoxanthine, mutations in the gene *cnx,* which encodes for the molybdenum cofactor, were ruled out, and the mutants unable to grow in nitrate were characterized as *niaD*$^{-}$.

A molecular characterization of the 21 *niaD*$^{-}$ mutants was carried out (Fig. 5). Three of these mutants presented insertion or deletion, whereas the others presented small mutations that did not alter the fragment size visually. Mutants PG3 and PG17 presented the insertion of a DNA segment of at least 2.4 kb within the fragment of 0.95-kb *Acc*I, which provides a smaller migration of this fragment (Fig. 5B). Besides, the mutant PG17 does not show the 1.8-kb *Acc*I fragment, revealing a different and more complex rearrangement than the mutant PG3. The mutation of strain PG53 seems to be punctual, without rearrangements, since its restriction pattern is identical to the wild type. Mutant PG63 presented

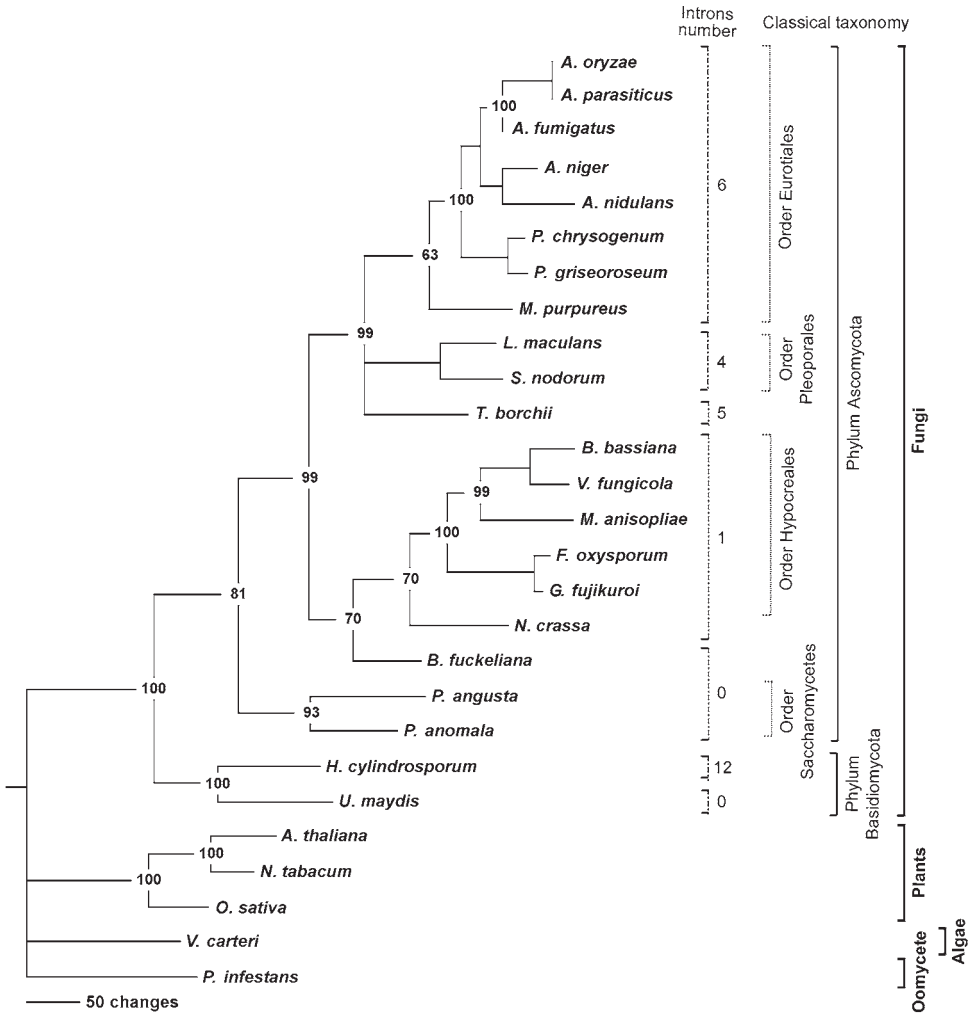
Fig. 3. Transcriptional analysis of the *Penicillium griseoroseum niaD* gene by RT–PCR. 0, 15, and 60 min indicate the period of exposure to nitrate or to glutamine after 36 h of growth under repression and activation conditions, respectively. Ammonium and urea indicate 36 h of growth under repressive conditions. A 0.5-kb γ-actin fragment (γ-act) was amplified as internal control.



a greater migration of the 0.95-kb *Acc*I fragments as a result of a deletion in this region.

The DNA fragment containing the deleted *niaD* gene region of the PG63 mutant was amplified and sequenced. This region presents a 122-bp deletion, corresponding to nucleotides from +2192 to +2313 based on the first proposed ATG. This sequence is responsible for coding 42 of the first 56 amino acids belonging to the FAD-binding domain. The deletion interrupts the codon to residue Lis^{616} and reaches to residue Gln^{656}. This region presents the residue His^{650}, which corresponds to the His^{654} of *Aspergillus nidulans*, whose importance for the functionality of the protein was demonstrated by Garde et al. (1995). Besides, there is a change in the open reading frame as well as the appearance of a stop codon (TAA) 12 bp after the deletion. Theo-

Fig. 4. Phylogram based on the parsimony analysis of nitrate reductase protein from fungi, plants, algae, and an oomycete. Horizontal distance is based on the number of inferred substitutions (parsimony criteria) and the vertical distance is arbitrary. Numbers indicate the percentage of bootstrap replicates from a sample of 1000 that support the branches in the percentage; values below 50% are not shown.



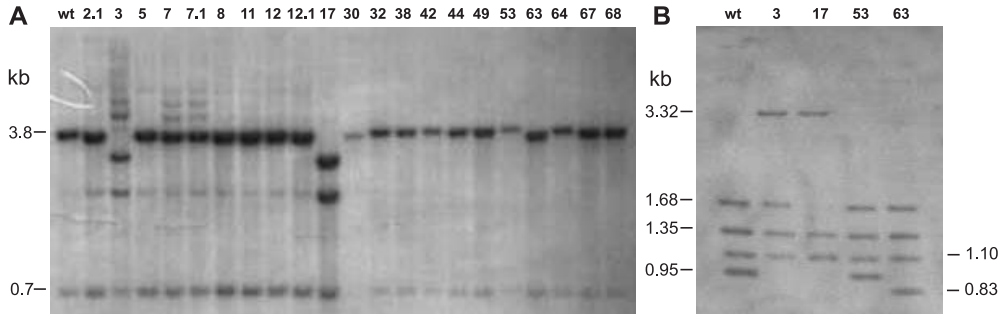
retically, this would lead to the formation of a 610 amino acid protein and a FAD-binding domain that presents only 15 amino acids.

The mutants PG4 and PG18, which failed to grow in nitrate and nitrite, were transformed with the *nirA* gene of *Aspergillus nidulans* to verify the presence of *nirA* gene mutations. These mutants were chosen, since they present a reversion frequency of less than 1×10^{-8}. Transformation frequency was about 9 transformants/µg DNA. About 10% of the transformants were abortive. Visually, the transformants presented normal growth and sporulation and did not differ in morphology when compared with the wild type.

Homologous transformation

To establish the homologous transformation system, the pNPG1 vector was used to complement the PG63 mutant. A transformation frequency of 14 transformants/µg DNA was obtained and no abortive transformants were visualized. After monosporic purification, 5 randomly homologous transformants were chosen to analyze the integration event. Total DNA of these transformants cleaved with *Sph*I, which does not cut the pNPG1 vector. Therefore, there is visualized in the *Penicillium griseoroseum* wild type a 6.7-kb fragment that contains the entire *niaD* gene (Fig. 6A.1). Because of this fragment's great size, the difference between the wild type and deleted *niaD* gene (122 bp) is not easily visualized in the electrophoresis. Two transformants (T1 and T5) analyzed showed a smaller migration of the *niaD* gene, indicating a homologous integration of the transformant vector (Fig. 6A.1). The difference in the size of the fragment between the wild type and these transformants is about 9 kb, which is the result of the integration of 1 copy of the transformation vector (Fig. 6B). To prove this integration type, the pBluescript vector was used to probe the transformants' DNA (Fig. 6A.2). These 2 transformants showed a hybridization of the same 15.7-kb fragment, whereas the wild type, PG63 mutant, and others transformants did not hybridize with the pBluescript vector. Besides not showing hybridization sign with the pBluescript vector, the transformants T2, T3, and T4 showed a hybridization pattern similar to that of

Fig. 5. Molecular analysis of *Penicillium griseoroseum niaD*⁻ mutants. (A) Hybridization of total DNA cleaved with *Eco*RV. The wild type (wt) presents 2 bands of approximately 3.8 and 0.7 kb. The numbers in each lane refer to mutant identification. (B) Hybridization of total DNA cleaved with *Acc*I of mutants PG3, PG17, PG53, and PG63, which presented possible rearrangements. The wild type presents 4 bands of approximately, 1.68, 1.35, 1.10, and 0.95 kb.

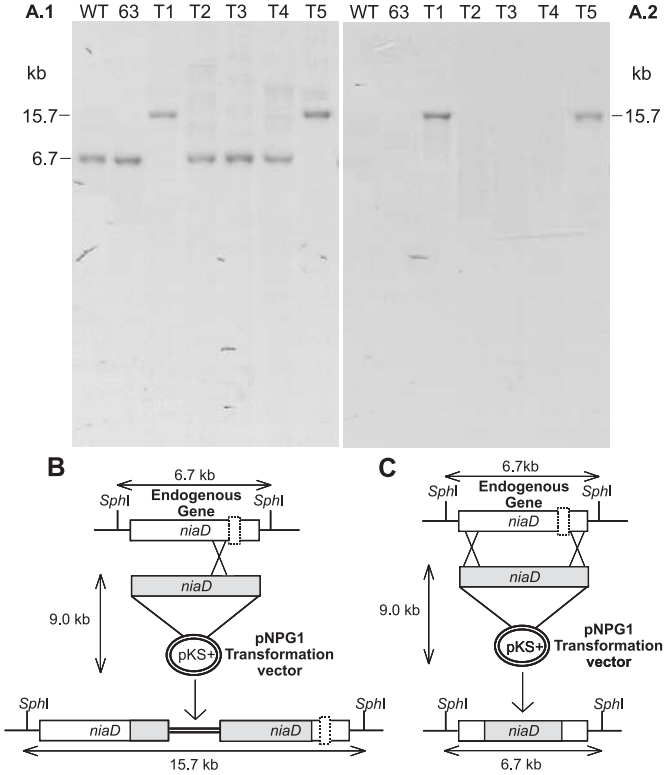


the wild type, indicating the occurrence of gene replacement (Fig. 6C).

Discussion

The promoter region of the *Penicillium griseoroseum niaD* gene presented sites to bind the general regulatory protein AREA and the protein NIRA. Elements with at least 2 GATA sequences, in the same or opposite direction and with spaces of at least 30 bp, are considered strong binding sites for NIT-2 (Chiang and Marzluf 1994). For *Penicillium chrysogenum*, strong binding sites for NRE were described by Haas and Marzluf (1995) as regions with at least 2 GATA sequences with spacing of between 5 and 27 bp aligned in the same or opposite direction. In the *Penicillium griseoroseum niaD* gene, 2 GATA sequences in the same direction, localized at –387 and –408 bp, are found at a distance of 17 bp, being able to offer a strong binding site for the general regulatory protein of nitrogen assimilation. Two other sequences in opposite directions, located at –38 and –89 bp, are at a distance of 47 bp and can perhaps also serve as a site for strong binding of general regulatory proteins. Intriguingly, the GATA sequence at –387 bp is linked to another sequence in the opposite direction. As in *Fusarium oxysporum* and *Penicillium chrysogenum*, only 1 binding site for the NIRA protein was found at –483 bp of the *niaD* promoter gene of *Penicillium griseoroseum*. As described by Punt et al. (1995), in a number of ascomycetes, there are potential NIRA-binding sites in *niaD* and (or) *niiA* isofunctional genes. Moreover, since the *Aspergillus nidulans* NIRA protein complemented *Penicillium griseoroseum nirA* mutants, it becomes clear that the *niaD* gene has a site that follows the *Aspergillus nidulans* consensus model. The similarity for the promoter region of *Penicillium griseoroseum* and *Penicillium chrysogenum* was 12.5%. Species within a same genus are expected to present high homology in the gene structural region but little or no similarity in the regulatory region. No similarity in the promoter region of the *niaD* gene is reported between *Aspergillus nidulans* and *Aspergillus niger* (Unkles et al. 1992), except for the regulatory-protein-binding sites. However, the similarity of the promoter region of *Penicillium griseoroseum* and *Penicillium chrysogeum* is relatively low, considering an identity of 42% in the nitrate and nitrite reductase intergenic region of *Aspergillus fumigatus* and *Aspergillus nidulans* and 37% in *Aspergillus*

Fig. 6. Hybridization analysis of *Penicillium griseoroseum* homologous transformants. WT, wild-type (63) *niaD*⁻ mutant used as recipient strain in transformation experiments; T1–T5, analyzed *niaD*⁺ transformants. Total DNA (3 µg) was digested with *Sph*I and probed with (A.1) pUES3.2 plasmid and (A.2) pBluescript vector. (B) Transformants T1 and T5 presented recombination in a homologous site involving 1 crossover; (C) transformants T2, T3, and T4 presented homologous recombination involving 2 crossovers (gene replacement).



parasiticus and the *nit-3* promoter region of *Neurospora crassa* (Amaar and Moore 1998). High identity values of 95% are reported for the corresponding regions of *Aspergillus parasiticus* and *Aspergillus oryzae* (Chang et al. 1996) and 91.2% for *Gibberella fujikuroi* and *Fusarium oxysporum* (Tudzynski et al. 1996).

The number and size of possibly introns present in the *Penicillium griseoroseum niaD* gene are similar to the ones

reported for other species, such as *Aspergillus nidulans*, whose introns vary from 48 to 67 bp (Johnstone et al. 1990), and *Penicillium chrysogenum*, with a variation of 50–71 bp (Haas et al. 1996). There is a similarity in the number of introns in *Aspergillus nidulans* (Johnstone et al. 1990), *Aspergillus niger* (Unkles et al. 1992), *Aspergillus oryzae* (Kitamoto et al. 1995), and *Penicillium chrysogenum* (Haas et al. 1996). The intron localization is the same for all of these species. Introns I and V of *Penicillium griseoroseum* presented similarity to those of *Penicillium chrysogenum*. No similarities among introns are reported in other species, even when they belong to the same genus, such as *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus oryzae* (Johnstone et al. 1990; Unkles et al. 1992; Kitamoto et al. 1995).

Expression analyses of the *niaD* gene reveal a highly efficiency response to nutritional variation in the environment. A fast response to such variations is also reported for *Neurospora crassa* (Okamoto et al. 1991) and *Penicillium chrysogenum* (Haas et al. 1996). For *Penicillium griseoroseum*, the *niaD* mRNA level was significantly reduced after 15 min of exposure to glutamine, revealing a mechanism that allows the cell to respond quickly to nutritional medium conditions and guarantees great energy savings. This is the first report on regulation analysis of the nitrate reductase gene using the RT–PCR technique. In contrast with most reports, a weak amplification signal of *niaD* cDNA was verified in total RNA extracted from mycelia grown in ammonium, urea, and glutamine. Owing to the greater detection sensibility of this technique, there is perhaps a basal expression of these genes.

The phylogenetic tree constructed using the NIAD protein amino acid sequence clustered closely related species in accordance to their possibly natural history. Williams et al. (1994) and Cutler et al. (1998) clustered some species of filamentous fungi showing the clustering of the basidiomycetes and ascomycetes and the separation of the oomycete *Phytophthora infestans*. It appears that the oomycetes are related to heterokont algae with chlorophylls *a* and *c* (Alexopoulos et al. 1996). Based on the analysis of this study, *Phytophthora infestans* is closer to the algae *V. carteri* than to filamentous fungi or yeasts. In a study of molecular evolution of nitrate reductase genes realized by Zhou and Kleinhofs (1996), the divergence time between fungi and plants was estimated to be 1000 million years. Cutler et al. (1998) discussed group agreement in cluster analysis with the number of introns in the *niaD* gene. In our work, this tendency was confirmed in Phylum Ascomycota but appears to be not true for Phylum Basidiomycota. This may be a reflex of the low number of *niaD* genes characterized in the basidiomycetes. Based on intron number and positions in the nitrate reductase genes of filamentous fungi, algae, and plants, Zhou and Kleinhofs (1996) could not affirm whether the introns were incorporated (gain hypothesis) or lost (loss hypothesis) during the evolution. The hypothesis of a shuffling exon was ruled out in this case, since the sequences that encode the functional domains of the nitrate reductase proteins are not separated by introns. Consequently, the introns for the genes that encode nitrate reductase might have been inserted after the divergence between fungi and plants, or the ancestor of these groups could have possessed all introns, which may have been lost independently after the divergence. The tendency to cluster the filamentous fungi based on order of the classical taxonomy represents a concrete scenario to use this sequence in filogenetics study of these organisms. Thus, even representing a small part of the genome, the *niaD* gene has conditions to separate species with a close phylogenetic relationship.

The chlorate-resistant mutants obtained in this study presented rearrangements with insertions and deletions. Insertions of DNA sequences into the nitrate reductase gene of various filamentous fungus species have been related to the presence of transposable elements (Daboussi 1997). The observed size of the insertion can be related to the reported size of some transposons, such as the element Flipper of *Botrytis cinerea* with 1.8 kb (Levis et al. 1997*b*) or the elements Fot1 and Fot2 of *Fusarium oxysporum* with 1.9 and 2.1 kb, respectively (Daboussi et al. 1992; Daboussi and Langin 1994), both inserted into the nitrate reductase gene. More research is necessary, however, to discriminate this insertion type from other recombination mechanisms. Mutant PG63, carrying a 122-bp deletion, presents a very interesting feature for its use as host strain in transformation experiments, since deletion is considered to be a mutation type without probability of reversion. This would guarantee that colonies grown in selective medium after transformation are really transformants and not revertants. Besides, the deleted segment size is small, which may provide a large contact region between the resident *niaD* gene and the transformation vector for possible homologous integration events.

The complementation of *nirA* mutations in *Penicillium* by the *Aspergillus nidulans nirA* gene is reported here for the first time. After transformation with plasmid pNIRA2, *Fusarium oxysporum nirA* mutants succeeded in growing in nitrate (Daboussi et al. 1991). These authors reported that transformant strains generally produced less mycelia in medium with nitrate as sole nitrogen source and presented a different morphology compared with the wild type. Morphological differences were not observed in *Penicillium griseoroseum nirA*$^+$ transformants. The isofunctionality of the specific regulatory protein in species of different genera shows that the nitrate metabolism seems to be conserved among ascomycetes.

The transformation frequency obtained with the *niaD* homologous transformation system (14 transformants/μg DNA) was higher than the heterologous transformation system (8 transformants/μg DNA) described by Queiroz et al. (1998). Although some articles using homologous transformation systems report a great increase in transformation frequency (Sànchez-Fernàndez et al. 1991; Tudzynski et al. 1996), for other species, that increase was moderated (Whitehead et al. 1990; Gouka et al. 1991; Levis et al. 1997*a*). The difference verified for *Penicillium griseoroseum* has not been promoted by the size of the vectors (approximately 9 kb for pNPG1 and 9.7 kb for pNE24). Among the 5 analyzed transformants, 2 presented the integration of 1 copy of the vector in the *niaD* locus and 3 presented events of gene replacement. These results reveal an increase in the rate of homologous integration when compared with the heterologous system, in which the analysis of 5 transformants demonstrated heterologous integration in 4 cases (Queiroz et al. 1998). For *Aspergillus oryzae* (Unkles et al. 1989) and *Stagonospora nodorum*

(Cutler et al. 1998), all the integrations occurred in the *niaD* locus. For *Botrytis cinerea* (Levis et al. 1997*a*), the analysis of 36 transformants revealed 34 homologous integrations. However, for filamentous fungi and other eukaryotic organisms, events of heterologous integration seem to be more common. Homologous transformation systems developed for *Cephalosporium acremonium* (Whitehead et al. 1990) and *Penicillium chrysogenum* (Gouka et al. 1991) revealed that, in most transformants, the vector integrates in locations different from the nitrate redutase locus. The observation of a high incidence of homologous integration in tha *Penicillium griseoroseum niaD* gene and other species does not imply a total integration directing in the *niaD* locus. However, for *Aspergillus nidulans*, studies of homologous transformation using the *niaD* and *amdS* (coding the enzyme acetamidase) genes reveal that the homologous integration of the *niaD* gene is at least 5 times higher than that of the *amdS* gene (Bird and Bradshaw 1997). According to those authors, it seems evident that there is variation in the efficiency of homologous integration between 2 loci of the same organism. A transformation system with high incidence of homologous integration, especially with 2 crossovers, can be used for studies of important areas of regulation and structural levels. Thus, mutations generated in vitro can be introduced and analyzed in the exact location where the gene resides. In addition, the rate of homologous integration is interesting in cotransformation experiments, lowering the probability of integrations in heterologous sites that are important for cell metabolism and (or) of biotechnological interest.

Acknowledgements

We thank the Brazilian agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support.

References

Alexopoulos, C.J., Mims, C.W., and Blackwell, M. 1996. Introductory mycology. John Wiley & Sons, Inc., New York.

Amaar, Y.G., and Moore, M.M. 1998. Mapping of the nitrate-assimilation gene cluster (*crnA–niiA–niaD*) and characterization of the nitrite reductase gene (*niiA*) in the opportunistic fungal pathogen *Aspergillus fumigatus*. Curr. Genet. **33**: 206–215.

Ballance, D.J., and Turner, G. 1985. Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene, **36**: 321–331.

Banks, G.R., Shelton, P.A., Kanuga, N., Holden, D.W., and Spanos, S. 1993. The *Ustilago maydis nar1* gene enconding nitrate reductase activity: sequence and transcriptional regulation. Gene, **131**: 69–78.

Bird, D., and Bradshaw, R. 1997. Gene targeting is locus dependent in the filamentous fungus *Aspergillus nidulans*. Mol. Gen. Genet. **255**: 219–225.

Burger, G., Tilburn, J., and Scazzocchio, C. 1991. Molecular cloning and functional characterization of the pathway-specific regulatory gene *nirA*, which controls nitrate assimilation in *Aspergillus nidulans*. Mol. Cell. Biol. **11**: 795–802.

Caddick, M.X., Peters, D., and Platt, A. 1994. Nitrogen regulation in fungi. Antonie Leeuwenhoek, **64**: 169–177.

Chang, P.K., Ehrlich, K.C., Linz, J.E.L., Bhatnagar, D., Cleveland, T.E., and Bennet, J.W. 1996. Characterization of the *Aspergillus parasiticus niaD* and *niiA* gene cluster. Curr. Genet. **30**: 68–75.

Chiang, T.Y., and Marzluf, G.A. 1994. DNA recognition by the NIT2 nitrogen regulatory protein: importance of the number, spacing, and orientation of GATA core elements and their flanking sequences upon NIT2 binding. Biochemistry, **33**: 576–582.

Cove, D.J. 1979. Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. **54**: 291–327.

Cutler, S.B., Cooley, R.N., and Caten, C.E. 1998. Cloning of the nitrate reductase gene of *Stagonospora* (*Septoria*) *nodorum* and its use a selectable marker for targeted transformation. Curr. Genet. **34**: 128–137.

Daboussi, M.J. 1997. Fungal transposable elements and genome evolution. Genetica, **100**: 253–260.

Daboussi, M.J., and Langin, T. 1994. Transposable elements in the fungal plant pathogen *Fusarium oxysporum*. Genetica, **93**: 49–59.

Daboussi, M.J., Langin, T., Deschamps, F., Brygoo, Y., Scazzocchio, C., and Burger, G. 1991. Heterologous expression of the *Aspergillus nidulans* regulatory gene *nirA* in *Fusarium oxysporum*. Gene, **109**: 155–160.

Daboussi, M.J., Langin, T., and Brygoo, Y. 1992. Fot1, a new family of fungal transposable elements. Mol. Gen. Genet. **232**: 12–16.

Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Binastein, M., and Goldberg, R.F. 1977. Primary activation of the vitellogenin gene in the rooster. J. Biol. Chem. **252**: 8310–8319.

Garde, J., Kinghorn, J.R., and Tomsett, A.B. 1995. Site-direct mutagenesis of nitrate reductase from *Aspergillus nidulans*. J. Biol. Chem. **270**: 6644–6650.

Gouka, R.J., Hartingsveldt, W.V., Bovenberg, R.A.L., Hondel, C.A.M.J.J., and Gorcom, R.F.M. 1991. Cloning of nitrate – nitrite reductase gene cluster of *Penicillium chrysogenum* and use of the *niaD* gene as a homologous selection marker. J. Biotechnol. **20**: 189–200.

Gurr, S.J., Unkles, S.E., and Kinghorn, J.R. 1987. The structure and organization of nuclear genes of filamentous fungi. *In* Gene structure in ukaryotic microbes. *Edited by* J.R. Kinghorn. IRL Press, Oxford, UK. pp. 93–139.

Haas, H., and Marzluf, G.A. 1995. NRE, the major nitrogen regulatory protein of *Penicillium chrysogenum*, binds specifically to elements in the intergenic promoter regions of nitrate assimilation and penicillin biosynthetic gene clusters. Curr. Genet. **28**: 177–183.

Haas, H., Marx, F., Graessle, S., and Stöffler, G. 1996. Sequence analysis and expression of the *Penicillium chrysogenum* nitrate reductase enconding gene (niaD). Biochim. Biophys. Acta, **1309**: 81–84.

Jargeat, P., Gay, G., Debaud, J.C., and Marmeisse, R. 2000. Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate. Mol. Gen. Genet. **263**: 948–956.

Jargeat, P., Rekangalt, D., Verner, M.C., Gay, G., Debaud, J.C., Marmeisse, R., and Fraissinet-Tachet, L. 2003. Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr. Genet. **43**: 199–205.

Johnstone, I.L., McCabe, P.C., Greaves, P., Gurr, S.J., Cole, G.E., Brow, M.A.D., Unkles, S.E., Clutterbuck, A.J., Kinghorn, J.R., and Innis, M.A. 1990. Isolation and characterisation of the

crnA–niiA–niaD gene cluster for nitrate assimilation in *Aspergillus nidulans*. Gene, **90**: 181–192.

Kitamoto, N., Kimura, T., Kito, Y., Ohmita, K., and Tsukagoshi, N. 1995. The nitrate reductase gene from a shoyu koji mold, *Aspergillus oryzae* KBN616. Biosci. Biotechnol. Biochem. **59**: 1795–1797.

Kudla, B., Caddick, M.X., Langdon, T., Martinez-Rossi, N.M., Bennett, C.F., Sibley, S., Davis, R.W., and Arst, H.N., Jr. 1990. The regulatory gene areA mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. **9**: 1355–1364.

Langdon, T., Sheerins, A., Ravagnani, A., Caddick, M.X., and Arst, H.N., Jr. 1995. Mutational analysis reveals dispensability of the N-terminal region of the *Aspergillus nidulans* transcriptional factor mediating nitrogen metabolite repression. Mol. Microbiol. **17**: 877–888.

Levis, C., Fortini, D., and Brygoo, Y. 1997*a*. Transformation of *Botrytis cinerea* with the nitrate reductase (niaD) shows a high frequency of homologous recombination. Curr. Genet. **32**: 157–162.

Levis, C., Fortini, D., and Brygoo, Y. 1997*b*. Flipper, a mobile Fot1-like transposable element in *Botrytis cinerea*. Mol. Gen. Genet. **254**: 674–680.

Marzluf, G.A. 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. **61**: 17–32.

Okamoto, P.M., Fu, Y.H., and Marzluf, G.A. 1991. *Nit*-3, the structural gene of nitrate reductase in *Neurospora crassa*: nucleotide sequence and regulation of mRNA synthesis and turnover. Mol. Gen. Genet. **227**: 213–223.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., MacDonald, K.D., and Bufton, A.W.J. 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. **5**: 141–238.

Punt, J.P., Strauss, J., Smit, R., Kinghorn, J.R., Van den Hondel, C.A.M.J.J., and Scazzochio, C. 1995. The intergenic region between the divergently transcribed *niiA* and *niaD* genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. Mol. Cell. Biol. **15**: 5688–5699.

Queiroz, M.V., Barros, A.O., Barros, E.G., Guimarães, W.V., and Araújo, E.F. 1998. Transformation of *Penicillium griseoroseum* nitrate reductase mutant with the *nia* gene from *Fusarium oxysporum*. Can. J. Microbiol. **44**: 1–3.

Ribon, A.O.B., Coelho, J.L.C., Barros, E.G., and Araújo, E.F. 1999. Cloning and characterization of a gene enconding the endopolygalacturonase of *Penicillium griseoroseum*. Biotechnol. Lett. **21**: 395–399.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

Sànchez-Fernàndez, R., Unkles, S.E., Campbell, E.I., Macro, J.A., Cerdà-Olmedo, E., and Kinghorn, J.R. 1991. Transformation of the filamentous fungus *Gibberella fujikuroi* using the *Aspergillus niger niaD* gene enconding nitrate reductase. Mol. Gen. Genet. **225**: 231–233.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, **74**: 5463–5467.

Specht, C.A., Dirusso, C.C., Novotny, C.P., and Ullrich, R.C. 1982. A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. Anal. Biochem. **119**: 158–163.

Swofford, D.L. 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Mass.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensibility of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673–4680.

Tudzynski, B., Mende, K., Weltring, K.M., Kinghorn, J.R., and Unkles, S.E. 1996. The *Gibberella fujikuroi niaD* gene enconding nitrate reductase: isolation, sequence, homologous transformation and electrophoretic karyotype location. Microbiology, **142**: 553–539.

Unkles, S.E., Campbell, E.I., Rtuiter-Jacobs, Y.M.J.T., Brockhuijsen, M., Macro, J.A., Carrez, D., Contreras, R., Van den Hondel, C.A.M.J.J., and Kinghorn, J.R. 1989. The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate reductase assimilation pathway: a convenient and general selection system for filamentous fungal transformation. Mol. Gen. Genet. **218**: 99–104.

Unkles, S.E., Campbell, E.I., Punt, P.J., Hawer, K.L., Contreras, R., Hawins, A.R., Van den Hondel, C.A.M.J.J., and Kinghorn, J.R. 1992. The *Aspergillus niger niaD* gene enconding nitrate reductase: upstream nucleotide and amino acid sequence comparisons. Gene, **111**: 149–155.

Whitehead, M.P., Gurr, S.J., Grieve, C., Unkles, S.E., Spence, D., Ramsden, M., and Kinghorn, R. 1990. Homologous transformation of *Cephalosporium acremonium* with the nitrate reductase-enconding gene (*niaD*). Gene, **90**: 193–198.

Williams, R.S.B., Davis, M.A., and Howlett, B.J. 1994. Nitrate reductase of the ascomycetous fungus, *Leptosphaeria maculans*: gene sequence and chromosomal location. Mol. Gen. Genet. **244**: 1–8.

Xiao, X., Fu, Y.H., and Marzluf, G.A. 1995. The negative-acting NMR regulatory protein of *Neurospora crassa* binds to and inhibits the DNA-binding activity of the positive-acting nitrogen regulatory protein NIT2. Biochemistry, **34**: 8861–8868.

Yelton, M.M., Hamer, J.E., and Timberlake, W.E. 1984. Transformation of *Aspergillus niger* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. USA, **81**: 1470–1474.

Zhou, J., and Kleinhofs, A. 1996. Molecular evolution of nitrate reductase genes. J. Mol. Evol. **42**: 432–442.